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Anti-bacterial activity of peritoneal cells from transgenic mice producing high levels of GM-CSF

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SUMMARY

Two lines of transgenic mice carrying the gene for granulocyte–macrophage colony-stimulating factor (GM-CSF) produce vastly increased numbers of macrophages with abundant foamy cytoplasm resembling classical activated macrophages. Cells from both lines were negative for myeloperoxidase, a bactericidal enzyme found in monocytes as well as neutrophils, but not mature macrophages. Cells from the so called 'male line' produced greatly increased levels of oxygen degradation products in response to phagocytosis, compared with cells from the 'female line' or from normal littermates. The ability of the cells to phagocytose and lyse the intracellular bacterium *Listeria monocytogenes* was tested *in vitro* using radiolabelled organisms. Although the cells from transgenic mice were more highly phagocytic than cells from normal littermates, cells from either line were no more efficient than normal at lysing the bacteria they had phagocytosed. Nevertheless, because of the high phagocytic rate, more bacteria were exposed to lysis in the cells of transgenic mice, and the final outcome was a higher rate of bacteriolysis.

INTRODUCTION

The haemopoietic growth and differentiation factors, known as colony-stimulating factors (CSF), are produced in increased amounts during infection (Quesenbury *et al.*, 1978; Trudgett *et al.*, 1973; Wing *et al.*, 1984; Young & Cheers, 1986). Since they have been shown *in vitro* to influence the activity of macrophages against various intracellular organisms (Handman & Burgess, 1979; Karbassi *et al.*, 1987; Lee & Warren, 1987), they are attractive candidates for therapy against these pathogens.

Listeria monocytogenes has been widely studied as an example of an intracellular bacterial pathogen which survives within normal macrophages but is readily destroyed by 'activated' macrophages (North, 1978). We have shown that experimental infection of mice with *L. monocytogenes* increases production of macrophage (M)-CSF, granulocyte (G)-CSF, and granulocyte–macrophage (G–M)-CSF (Cheers *et al.*, 1988; Cheers & Stanley, 1988). Furthermore, 24 hr exposure *in vitro* of peritoneal macrophages to M-CSF increases their overall bactericidal activity (Cheers *et al.*, 1989).

The construction of two lines of transgenic mice which continuously express excessive levels of GM-CSF *in vivo* (Lang *et al.*, 1987) gave us the opportunity to test the activity of their peritoneal cells against *L. monocytogenes*. The two lines are named after their original progenitors, one, the male line, carrying the transgene autosomally, the other, the female line, carrying the transgene on the x chromosome. Both lines produce vastly increased numbers of macrophages which invade the tissues, in particular the peritoneal cavity, and which have been shown to be highly phagocytic for erythrocytes (Lang *et al.*, 1987). The peritoneal macrophages from the male line have an abundant cytoplasm with foamy appearance, similar to the 'activated' macrophages observed during infection (Tran *et al.*, 1990), while those from the female line have less cytoplasm but stain strongly basophilic (Metcalf & Moore, 1988). Nor is appearance of the cells the only way in which the two strains differ. The male line produces more macrophages and more interleukin-1 (IL-1), presumably as a result of stimulation of the macrophages by GM-CSF (Gearing *et al.*, 1989). The disease pattern in the transgenic mice which results from invasion of their tissues by macrophages and leads to their early death, also differs in the two lines (Metcalf & Moore, 1988).

We therefore undertook an analysis of the anti-bacterial activity of cells from each line in comparison with their normal littermates. We show here that the cells from transgenic mice are very efficient at phagocytosing *Listeria* organisms and that the bacteria are thus exposed to intracellular killing. However, despite higher than normal generation of oxygen degradation products in the male line, cells from neither line were more efficient than normal at lysing the engulfed bacteria.

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MATERIALS AND METHODS

Mice

The transgenic mice bred and maintained under specific pathogen-free conditions at the Walter and Eliza Hall Institute, Australia, and carrying the continuously expressed GM-CSF gene, have been described in detail elsewhere (Lang *et al.*, 1987). These mice, and their non-transgenic littermates, were (SJL × C57BL/6) F_{12}. Both parent strains are genetically resistant to *Listeria* (Cheers & McKenzie, 1978). Other mice used were C57BL/10, also genetically resistant to *Listeria* and bred and maintained under conventional but disease-free conditions at the Department of Microbiology, University of Melbourne, Australia. Female mice only were used at age 8 weeks or more.

Peritoneal cells

Mice were given a lethal dose of fluothane anaesthetic, and cells from their peritoneal cavity washed out with 5 ml HEPES-buffered Dulbecco's modified Eagle's medium (HEPES-DMEM) containing 10 U heparin/ml. The cells were washed by centrifuging at 800 ***g*** for 7 min through a foetal calf serum (FCS) cushion. They were resuspended in HEPES-DMEM or Hanks' balanced salt solution (HBSS) at a concentration suitable to the test(s) to be carried out. Where non-transgenic mice were used, the peritoneal cells from three mice were pooled for each experiment. Where transgenic mice were used, each experiment represents a single donor mouse. However, the experiments were repeated a number of times, as noted in the text, and the results were completely reproducible.

Myeloperoxidase stain

Cytocentrifuge preparations of peritoneal cells were fixed in formal-ethanol and stained with benzidine dihydrochloride mixture as described by Kaplow (1965). Cells with blue granules are positive for myeloperoxidase (MPO^+). Polymorphonuclear cells and mononuclear cells were distinguished by size and by the distribution of blue granules in the cytoplasm. Other cytocentrifuge preparations were stained by Diff Quik (Lab Aids, Narrabeen, NSW, Australia) for differential counts.

Superoxide anion (O_2^-) release

This test was modified from Rook *et al.* (1985) using cells in suspension. To each well of a flat-bottomed microtitre tray 2×10^5 viable cells in 50 μl HBSS were added followed by 100 μl of a 1 mg/ml nitroblue tetrazoleum (NBT) solution containing 100 μl/ml heat-killed yeasts (absorbance at 540 nm = 1·6). Absorbance at 620 nm was recorded using a Titertek Multiscan MC before and after 1 hr incubation at 37°, when reduced NBT was visible as blue deposits. Results were expressed as absorbance values ± standard deviation from eight replicates. Background absorbance recorded before incubation was subtracted.

Hydrogen peroxide (H_2O_2) release

This test was modified from Pick & Kesari (1980). To 50 μl of a cell suspension containing 2×10^5 viable cells in a flat-bottomed microtitre well, 100 μl of a Hanks' balanced salt solution containing 0·56 mM phenol red, 20 U/ml horseradish peroxidase and 100 μl/ml heat-killed yeast (absorbance at 540 nm = 1·6) were added. To each control well, 10 μl of 1 N NaOH were added immediately. The tray was then incubated at 37° for 90 min. Following incubation, 10 μl of 1 N NaOH were added to each of the remaining wells to terminate the reaction and absorbance at 620 nm recorded. Results were expressed as absorbance values ± standard deviation of eight replicates. Background absorbance from wells where the reaction was terminated before incubation was subtracted.

Phagocytosis and bacteriolytic assay

This assay was modified from Davies (1983), as previously described (Cheers *et al.*, 1989). Briefly, *Listeria* organisms were labelled metabolically at 25° for 16 hr with tritiated thymidine ([^{3}H]TdR) and approximately 4×10^7 bacteria (50,000 c.p.m.) were mixed with 2×10^6 viable peritoneal cells in 1 ml HEPES-DMEM. After 30 min at 37°, in some experiments the cells were washed free of unassociated bacteria by centrifuging at 100 ***g*** for 10 min at 4° through a cushion of FCS. Uptake of radioactivity was measured at that point, before a further 2 hr incubation and trichloracetic acid (TCA) precipitation. In this case, released TCA-soluble label, representing lysed bacteria, could be expressed as a percentage of uptake. In other instances the mixture of cells and bacteria was incubated for the full $2\frac{1}{2}$ hr before TCA precipitation, allowing a total assessment of phagocytosis and bacteriolysis. Release of TCA-soluble material in the absence of cells was subtracted in expressing the results. Where phagocytic activity was assessed microscopically, the bacteria were not radiolabelled but incubated with the cells at a ratio of 20:1. The mixture was sampled at intervals, 2×10^4 cells (10 μl) were layered onto 200 μl cold foetal calf serum in the well of a cytocentrifuge and the whole was spun at 60 ***g*** for 10 min.

RESULTS

Morphology of peritoneal cells from transgenic mice or their normal littermates

Unelicited peritoneal cells from either the male or female line of GM-CSF transgenic mice or from their normal littermates were compared in Diff Quik-stained smears (Table 1) and in smears stained for myeloperoxidase activity (Table 2). While both lines of transgenic mice produced vastly increased numbers of peritoneal cells compared with their normal littermates, the male line mouse produced even more than the female line mouse. This is also noted in other tables. Simple differential counts showed a much higher percentage of macrophages in the transgenic lines of mice than in their normal littermates (Table 1). The macrophages from the male line transgenic mice had abundant, foamy cytoplasm, similar to that seen in macrophages activated by infection, while macrophages from the female line had less cytoplasm and stained strongly basophilic. This difference was documented by Metcalf & Moore (1988). However, in contrast to the inflammatory macrophages elicited by intraperitoneal infection with *L. monocytogenes*, very few of the cells from either line of transgenics stained positive for myeloperoxidase activity (Table 2). It should be noted that the myeloperoxidase does not give sufficient resolution to divide the cells into macrophages and lymphocytes, or polymorphs (neutrophils) and eosinophils, hence they are bracketed in Table 1 under the term 'mononuclear cells' or 'polymorphonuclear cells'.

Table 1. Differential counts on peritoneal cells from representative individual transgenic mice and their normal littermates

Group	Total cells recovered per mouse	% cells				
		Polymorphs	Lymphocytes	Monocytes	Eosinophils	Mast cells
Male line transgenics						
Mouse 1	104×10^6	0	0	97	3	0
2	70×10^6	0	11	83	6	0
3	116×10^6	0	4	92	4	0
Female line transgenics						
Mouse 1	26×10^6	0	16	84	0	0
2	35×10^6	0	22	78	0	0
3	14×10^6	0	16	84	0	0
Littermates						
Pool 1	2×10^6	0	45	50	2	3
2	4×10^6	0	54	45	0	1
3	2×10^6	0	25	71	0	4
Inflammatory exudate*						
Pool 1	6×10^6	19	6	75	0	0

* Mice injected 2 days before harvesting with 1×10^6 *L. monocytogenes*.

Table 2. Myeloperoxidase activity of peritoneal cells from transgenic or normal mice*

Group	Myeloperoxidase stain		
	MPO^+ mononuclears	MPO^- mononuclears	Polymorphonuclear cells
Male line transgenics	4±1	90±1	6±1
Female line transgenics	3±1	95±1	3±1
Littermate controls	0	99±1	0·7±0·6
Inflammatory exudate†	33±9	48±8	20±4

* At least 100 sequential cells on each of three slides stained for myeloperoxidase (MPO) activity were counted. Figures represent percentage of total cells±SD. Mononuclear cells includes both macrophages and lymphocytes. Polymorphonuclear cells includes both polymorphonuclear neutrophils and eosinophils.

† Mice were injected 2 days before harvesting with 1×10^4 *L. monocytogenes*.

Table 3. Production of O_2^- metabolites by cells from transgenic mice or their littermates

Cell source*	O_2^- production† (absorbance at 620 nm)	H_2O_2 production† (absorbance at 620 nm)
Male line transgenics	0·070±0·016‡	0·092±0·018‡
Female line transgenics	0·022±0·009§	0·000±0·014§
Littermates	0·015±0·014	0·001±0·018
B10 peritoneal exudate¶	0·085±0·020	0·081±0·018
B10 resident cells	0·046±0·013	0·008±0·016

* Number of cells recovered from peritoneal cavity per mouse: male line transgenic, 104×10^6; female line transgenic, 26×10^6; littermates, 2×10^6.

† O_2^- and H_2O_2 release were measured using eight replicates and background absorbance subtracted as described in the Materials and Methods.

‡ $P < 0{\cdot}001$ compared with littermates and female line.

§ Not significantly different from littermates.

¶ C57BL/10 mice were injected i.p. 2 days earlier with 1×10^4 *L. monocytogenes* to provide a positive control.

Production of oxygen metabolites by cells from transgenic mice or their littermates

Production of superoxide anion (O_2) and H_2O_2 in response to a phagocytic stimulus (heat-killed yeast) was measured for the three cell populations (Table 3). The results shown are typical of five experiments representing different individual mice. The male line transgenics were very efficient at generating these products, which are sometimes claimed to be associated with bacterial killing, but the female line showed no significant increase over normal. As a positive control, conventional C57BL/10 mice were injected intraperitoneally with *L. monocytogenes*, and it may be seen that the production of oxygen degradation products by the resulting inflammatory population was of the same order of magnitude as that seen in the male-line macrophages.

Phagocytic and bacteriolytic activity of peritoneal macrophages from transgenic mice or their littermates

Peritoneal cells from either the male or female line of transgenic mice or from their normal littermates were mixed with a suspension of *L. monocytogenes* at a bacteria to cell ratio of 20:1

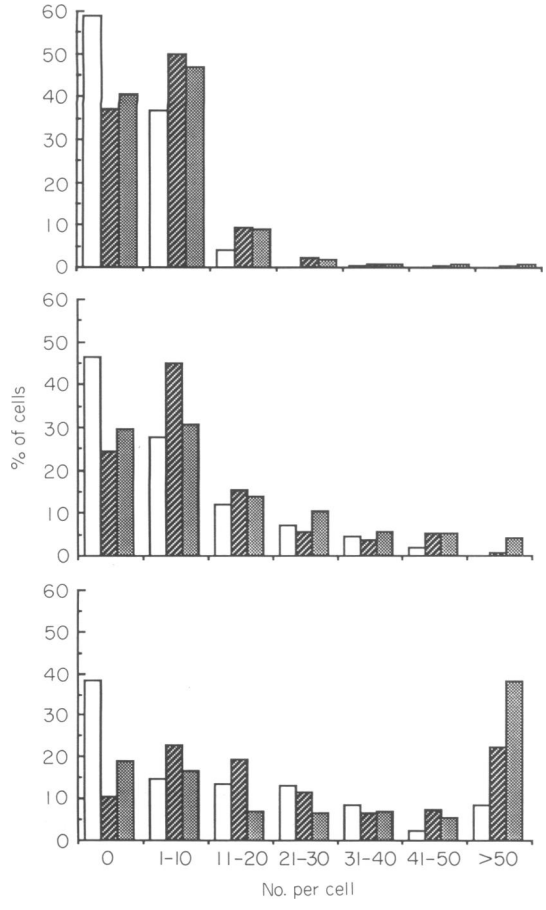

Figure 1. Numbers of bacteria per cell after 30 min (a), 60 min (b) and 120 min (c) phagocytosis by cells from male line GM-CSF transgenic mice (■), female line GM-CSF transgenic mice (▨) and their normal littermates (□).

and incubated in a 37° waterbath. At 30-min intervals the suspensions were sampled for cytocentrifuging. Cells with macrophage morphology from both transgenic lines were more efficiently phagocytic than those from the normal littermates in terms of the distribution of bacteria per cell (Fig. 1) as well as the number of phagocytic cells and the mean number of bacteria per cell (Table 4). When the latter two figures were combined in a phagocytic index, it may be seen that the macrophages from transgenic mice were about twice as efficient at phagocytosis compared with normal.

This efficiency of the cells from transgenic mice was confirmed when uptake of [^{3}H]TdR-labelled *L. monocytogenes* was measured after 30 min (Table 5). As a measure of bacteriolytic activity, the amount of soluble radioactivity released after a further 2 hr incubation was assayed. The cells from transgenic mice, as a result of their greater phagocytosis, released more soluble label than did littermate cells. However, when this release was expressed as a percentage of the radioactivity taken up by the cells, those from the transgenic mice were actually somewhat less efficient than normal. This is in contrast to the cells accumulating in the peritoneal cavity 2 days after injection of *Listeria* organisms. These showed increased efficiency of both phagocytosis and lysis. The results shown are typical of four experiments performed.

Table 4. Phagocytic activity of peritoneal macrophages from transgenic mice or their littermates*

Group†	Time of incubation (min)	% macrophages phagocytic	Bacteria per phagocytic cell	Phagocytic index‡
Male line transgenics	30	61±5	7·0±7·7	427
	60	75±2	13·3±13·6	998
	120	90±1	26·7±17·9	2403
Female line transgenics	30	58±4	7·5±8·1	435
	60	70±4	18·9±15·4	1323
	120	81±4	34·2±18·6	2770
Littermates	30	41±3	5·4±5·0	221
	60	53±3	14·6±11·9	774
	120	62±3	23·9±15·5	1481

* Cells and bacteria (1:20) were incubated in a 37° waterbath in quadruplicate and sampled at intervals for cytocentrifuging (see the Materials and Methods). At least 100 sequential cells on each replicate slide were counted at 1000× magnification.

† Number of cells recovered from peritoneal cavity per mouse: male line transgenices, 70×10^6; female line transgenices, 35×10^6; littermates, $4{\cdot}0 \times 10^6$.

‡ Product of percentage cells phagocytic and mean number of bacteria per phagocytic cell.

Table 5. Phagocytic and bacteriolytic activity of peritoneal cells from transgenic mice and their littermates

Group*	c.p.m. phagocytosed†	TCA-soluble c.p.m. released‡	% release§
Male line transgenics	4056±414	303±20	7·5
Female line transgenics	3860±250	370±22	9·6
Littermates	973±167	130±20	13·4
B10 peritoneal exudate¶	6855±277	1502±80	22
B10 resident cells	2464±353	236±40	9·6

* Number of cells recovered from peritoneal cavity per mouse: male line transgenic, 116×10^6; female line transgenic, 14×10^6; littermates, 2×10^6.

† Cells and bacteria (1:20) were incubated in quadruplicate at 37° for 30 min then washed free of unassociated bacteria as described in the Materials and Methods. Difference between two lines of transgenic mice not significant, but differences between them and littermates significant at $P < 0{\cdot}001$ by Student's *t*-test.

‡ After 30 min phagocytic period the cells were further incubated for 2 hr and amount of soluble ^{3}H released measured. Difference between two lines of transgenic mice not significant, but differences between them and littermates significant at $P < 0{\cdot}001$ by Student's t-test.

§ Percentage of phagocytosed radioactivity released as soluble label.

¶ C57BL/10 mice were injected i.p. with 1×10^4 *L. monocytogenes* 2 days earlier to provide a positive control.

Because this release of radiolabel compared the activities of the total populations, which differed in their percentage of macrophages, total bacteriolytic activity (the combined effect of phagocytosis and digestion) was tested at two different cell concentrations but with constant bacterial numbers (Table 6). It

Table 6. Anti-bacterial activity of peritoneal cells from transgenic mice compared with littermates*

Cell source†	Cell no.	c.p.m. released
Male line transgenics	4×10^6	340 ± 28
	2×10^6	248 ± 12
Female line transgenics	4×10^6	434 ± 30
	2×10^6	242 ± 22
Littermates	4×10^6	132 ± 18
	2×10^6	124 ± 32

* Cells and bacteria (1:20 or 1:10) were incubated in quadruplicate for $2\frac{1}{2}$ hr and total TCA-soluble radioactivity released was measured.

† Number of cells recovered from peritoneal cavity per mouse: male line transgenic, 178×10^6; female line transgenic, 5×10^6; littermates, 2×10^6.

can be seen that 2×10^6 cells from transgenic mice were more efficient than 4×10^6 from their normal littermates. This experiment was performed twice with similar results.

DISCUSSION

The numbers of peritoneal macrophages in the transgenic mice studied here are vastly increased by continuous exposure to high levels of GM-CSF (Lang *et al.*, 1987). They apparently do not represent newly formed cells but have the appearance of fully mature 'activated' macrophages.

Their status as mature macrophages rather than young monocytes is here confirmed by the absence of myeloperoxidase activity, as assessed by staining. This enzyme is found in both polymorphonuclear neutrophils and monocytes, but not mature macrophages (Kaplow, 1965) and presumably contributes to bactericidal activity (Babior, 1984). We have demonstrated myeloperoxidase in cells of the radiosensitive early (1–2 days) inflammatory response to *L. monocytogenes* (Tran *et al.*, 1990), which are remarkably similar in appearance to the macrophages from male line GM-CSF transgenic mice. The inflammatory macrophages in listeriosis are recently divided, as shown by [^{3}H]TdR labelling. Myeloperoxidase activity is lost later in infection, but the cells remain actively listericidal.

The present study confirmed the fact that, while there is variation among individual animals, the male line mice produce more macrophages than the female line. Furthermore, cells from the two transgenic lines differed in their ability to generate oxygen degradation products in response to phagocytosis of yeast particles, the male line being more active than the female line. It is not clear why the site of insertion of the GM-CSF gene should influence this or the other parameters which differ in the two lines (Metcalf & Moore, 1988; Gearing *et al.*, 1989). In H_2O_2 and O_2^- production, the cells from the male line resembled those activated by infection with *L. monocytogenes* (Tran *et al.*, 1990).

The anti-bacterial activity of the cells was tested by exposing them to radiolabelled *L. monocytogenes*. In this respect both lines showed a heightened phagocytic rate, judged microscopically or by associated radiolabel after a period of phagocytosis and careful washing. It is of course difficult to state, without specialized techniques, whether every cell-associated bacterium is intracellular, but evidence presented by Davies (1983) suggests that once *Listeria* organisms adhere to the cell surface, they are rapidly taken in and killed. Because of the high rate of phagocytosis by cells from the transgenic mice, bacteria exposed to them were lysed at a relatively high rate. However, when the process was broken down into its components, namely phagocytosis and bacteriolysis, and the release of TCA-soluble label (representing lysed bacteria) was expressed as a percentage of label taken up by the cells, then bacteriolysis by the cells from transgenic mice was revealed as no higher than normal. The failure of the GM-CSF to enhance bacteriolysis of *Listeria* organisms is unlikely to be due to the *Listeria* resistance of the parental strains, since genetic resistance to *Listeria* relates to the inflammatory response, not the bactericidal potential of the resident macrophages (Wood *et al.*, 1986).

The failure of the cells from transgenic mice to show increased lytic efficiency is of particular interest in the case of the male line of mice, where the increased generation of oxygen degradation products would lead many to predict that they would show increased bactericidal capacity (Hamilton & Adams, 1987). However, considerable doubt has been cast on the role of these products to kill actively growing *L. monocytogenes* (Bertolossi *et al.*, 1987) and this, taken together with the present results, calls for continued questioning of the role of oxygen degradation products in the killing of this and other organisms.

Finally it should be pointed out that another CSF, namely M-CSF, has also been tested for its ability to enhance antilisterial activity with rather similar results (Cheers *et al.*, 1989). When peritoneal macrophages were exposed *in vitro* for 24 hr to 1000 U/ml M-CSF, they developed increased phagocytic activity, without an increase in bacteriolytic activity *per se*. Nevertheless, in both instances, because more bacteria are exposed to intracellular killing as a result of 'activation' of macrophages by these two CSF, it is still likely that they represent possible candidates for therapy of bacterial infection.

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